BONE GRAFT AND SCAFFOLDING MATERIALS IMMOBILIZED WITH OSTEOGENESIS ENHANCING PEPTIDES ON THE SURFACE

TECHNICAL FIELD

The present invention relates to a bone graft material and a scaffold for tissue engineering applications, which have osteogenesis-promoting peptides immobilized on the surface, and more particularly, to a bone graft material and a scaffold for tissue engineering applications (hereinafter, referred to as scaffold), which have a cell adhesion-inducing peptide and/or tissue growth factor-derived peptide immobilized on the surface.

BACKGROUND ART

Periodontal tissue that supports teeth consists generally of the alveolar bone, the periodontal ligament tissue forming the periodontal membrane between the alveolar bone and the teeth, and the connective tissue. The loss of alveolar bone caused by the progression of periodontitis involves the loss of periodontal ligament tissue, and at sites with the loss of periodontal ligament tissue, the normal repair of alveolar bone and periodontal ligament tissue after the treatment of periodontitis becomes impossible due to excessive growth of connective tissue. Also, even when new bone is formed, the periodontal ligament tissue will not be normally differentiated so that the loss of tooth function can be caused.

To solve such problems, an attempt to induce the complete regeneration or new formation of tissue using an artificial barrier membrane together with autografting in guided periodontal regeneration is actively made. Also, for the regeneration of bone tissue, a tissue engineering scaffold is used as a bone graft material. Since cases showing the effective induction of periodontal tissue and bone tissue by the introduction of bone graft materials and scaffolds (Camelo, M. *et. al*, *International J. Periodont. Restorative Dent.* 21:109, 2001) were reported for recent ten years, various materials, including bone powder particles made of bovine bone, have been used as bone materials and tissue engineering scaffolds for tissue regeneration.

Meanwhile, in order to improve the efficiency of such bone graft materials and scaffolds for tissue regeneration, studies to attach materials capable of improving tissue regeneration to the bone graft materials and the scaffolds are now conducted. Among such materials, extracellular matrices or specific tissue growth factors are reported to be excellent in the ability of the repair and regeneration of damaged tissue, and their excellent ability to regenerate tissue was also shown in the results of actual clinical tests.

However, there have been drawbacks that most of the extracellular matrices and growth factors are relatively expensive and are unstable in high-molecular weight living bodies having a molecular weight of several tens kDa, leading to a reduction in the activity. Particularly, there have been problems that they disappear in a few minutes so that they should be administered at high dose to achieve the desired therapeutic effect and thus, cause side effects.

Recently, there have been attempts to reduce shortcomings with a simple application of these materials by adding the tissue growth factors to bone graft materials used in guided bone regeneration and polymer scaffolds used in tissue engineering so as to induce sustained release of the tissue growth factors.

Also, some effects of such attempts were proved. However, there is a disadvantage in that, with these bone graft materials or scaffolds themselves, the tissue growth factors are physically mixed, so that, in initial application, the burst release of the growth factors occur, thus making it difficult to maintain the tissue growth factors at an effective concentration for a treatment period.

DISCLOSURE OF INVENRION

Accordingly, the present inventors have made extensive efforts to solve the above-described problems occurring in the prior art, and consequently found that a bone graft material and scaffold having a surface immobilized with the active site peptides of a tissue growth factor and an extracellular matrix protein, which can achieve a tissue regeneration effect, show stable and lasting pharmacological effects, even when a low concentration dose level of the peptides are adhered thereto. On the basis of this finding, the present has been completed.

An object of the present invention is to provide a bone graft material and a scaffold for tissue engineering applications, which can achieve the desired tissue regeneration effect even with low concentration dose levels of cell adhesion-inducing peptide and/or tissue growth factor-derived peptide.

To achieve the above object, the present invention provides a bone graft material and a scaffold for tissue engineering applications, which have a cell adhesion-inducing peptide and/or tissue growth factor-derived peptide immobilized on the surface.

Specifically, the present invention provides a bone graft material and a scaffold,

on the surfaces of which a cell adhesion-inducing peptide and/or tissue growth factor-derived peptide having pharmacological activity are immobilized so that the bone graft material and the scaffold have pharmacological activity, whereby their efficiency for the regeneration of bone tissue or other tissues can be increased.

The cell adhesion-inducing peptide or the tissue growth factor-derived peptide is obtained by isolating and extracting the active-site amino acid sequence of physiologically active cytokine and subjecting the extract to chemical modification so as to maintain its active structure.

Specifically, the cell adhesion-inducing peptide is preferably a peptide having an amino acid sequence of RGD. More preferably, it is CGGRGDS (SEQ ID NO: 1) or CGGVACDCRGDCFC (SEQ ID NO: 2) designed to maintain the structural stability of the amino acid sequence of RGD.

Furthermore, the tissue growth factor-derived peptide is a peptide identified and chemically synthesized from the active site of the tissue growth factor. Preferably it is at least one peptide selected from the group consisting of the following peptides:

(a) the amino acid sequence at positions 2-18 of each of bone morphogenetic proteins (BMP)-2, 4 and 6 [SEQ ID NO: 3 for BMP-2, SEQ ID NO: 4 for BMP-4, and SEQ ID NO: 5 for BMP-6]; the amino acid sequence at positions 16-34 of BMP-2 (SEQ ID NO: 6), the amino acid sequence at positions 47-71 (SEQ ID NO: 7), the amino acid sequence at positions 73-92 (SEQ ID NO: 8), the amino acid sequence at positions 88-105 (SEQ ID NO: 9), the amino acid sequence at positions 283-302 (SEQ ID NO: 10), the amino acid sequence at positions 335-353 (SEQ ID NO: 11) and the amino acid sequence at positions 370-390 (SEQ ID NO: 12); the amino acid sequence at positions 74-93 of BMP-4 (SEQ ID NO: 13), the amino acid sequence at

positions 293-313 (SEQ ID NO: 14), the amino acid sequence at positions 360-379 (SEQ ID NO: 15) and the amino acid sequence at positions 382-402 (SEQ ID NO: 16); the amino acid sequence at positions 91-110 of BMP-6 (SEQ ID NO: 17), the amino acid sequence at positions 397-418 (SEQ ID NO: 18), the amino acid sequence at positions 472-490 (SEQ ID NO: 19) and the amino acid sequence at positions 487-510 (SEQ ID NO: 20); and the amino acid sequence at positions 98-117 of BMP-7 (SEQ ID NO: 21), the amino acid sequence at positions 320-340 (SEQ ID NO: 22), the amino acid sequence at positions 390-409 (SEQ ID NO: 23) and the amino acid sequence at positions 405-423 (SEQ ID NO: 24);

(b) the amino acid sequence at positions 62-69 of bone sialoprotein (SEQ ID NO: 25), the amino acid sequence at positions 139-148 (SEQ ID NO: 26), the amino acid sequence at positions 259-277 (SEQ ID NO: 27), the amino acid sequence at positions 199-204 (SEQ ID NO: 28), the amino acid sequence at positions 151-158 (SEQ ID NO: 29), the amino acid sequence at positions 275-291 (SEQ ID NO: 30), the amino acid sequence at positions 20-28 (SEQ ID NO: 31), the amino acid sequence at positions 65-90 (SEQ ID NO: 32), the amino acid sequence at positions 150-170 (SEQ ID NO: 33) and the amino acid sequence at positions 280-290 (SEQ ID NO: 34);

(c) the amino acid sequence at positions 242-250 of a transforming growth factor (SEQ ID NO: 35), the amino acid sequence at positions 279-299 (SEQ ID NO: 36) and the amino acid sequence at positions 343-361 (SEQ ID NO: 37);

(d) the amino acid sequence at positions 100-120 of a platelet-derived growth factor (SEQ ID NO: 37) and the amino acid sequence at positions 121-140 (SEQ ID NO: 39);

(e) the amino acid sequence at positions 23-31 of an acidic fibroblast growth factor (SEQ ID NO: 40) and the amino acid sequence at positions 97-105 (SEQ ID NO: 41);

(f) the amino acid sequence at positions 16-27 of a basic fibroblast

growth factor (SEQ ID NO: 42), the amino acid sequence at positions 37-42 (SEQ ID NO: 43), the amino acid sequence at positions 78-84 (SEQ ID NO: 44) and the amino acid sequence at positions 107-112 (SEQ ID NO: 45);

(g) the amino acid sequence at positions 255-275 of dentin sialoprotein (SEQ ID NO: 46), the amino acid sequence at positions 475-494 (SEQ ID NO: 47) and the amino acid sequence at positions 551-573 (SEQ ID NO: 48);

(h) the amino acid sequence at positions 63-83 of a heparin binding EGF-like growth factor (SEQ ID NO: 49), the amino acid sequence at positions 84-103 (SEQ ID NO: 50), the amino acid sequence at positions 104-116 (SEQ ID NO: 51) and the amino acid sequence at positions 121-140 (SEQ ID NO: 52);

(i) the amino acid sequence at positions 326-350 of the cadherin EGF LAG seven-pass G-type receptor 3 (SEQ ID NO: 53), the amino acid sequence at positions 351-371 (SEQ ID NO: 54), the amino acid sequence at positions 372-400 (SEQ ID NO: 55), the amino acid sequence at positions 401-423 (SEQ ID NO: 56), the amino acid sequence at positions 434-545 (SEQ ID NO: 57), the amino acid sequence at positions 546-651 (SEQ ID NO: 58), the amino acid sequence at positions 1375-1433 (SEQ ID NO: 59), the amino acid sequence at positions 1435-1471 (SEQ ID NO: 60), the amino acid sequence at positions 1475-1514 (SEQ ID NO: 61), the amino acid sequence at positions 1515-1719 (SEQ ID NO: 62), the amino acid sequence at positions 1764-1944 (SEQ ID NO: 63) and the amino acid sequence at positions 2096-2529 (SEQ ID NO: 64); and

(j) the amino acid sequence at positions 54-159 of an osteoblast specific cadherin (OB-cadherin) (SEQ ID NO: 65), the amino acid sequence at positions 160-268 (SEQ ID NO: 66), the amino acid sequence at positions 269-383 (SEQ ID NO: 67), the amino acid sequence at positions 384-486 (SEQ ID NO: 68) and the amino acid sequence at positions 487-612 (SEQ ID NO: 69).

More preferably, the N-terminal end of the peptide has an addition of a spacer

(CGG-) consisting of cysteine and two glycines so as to facilitate chemical immobilization of the peptide to the bone graft material and the scaffold.

The active peptides according to the present invention are obtained by synthesizing each of sequences of 10-20 amino acids in the total amino acid sequence of the tissue growth factor, subjecting the synthesized amino acid sequences to cell adhesion tests to select amino acid sequences having the highest activity, and subjecting the terminal end of the selected amino acid sequences to chemical modification so as to facilitate the immobilization of the modified amino acid sequences to the bone graft material and the scaffold. Thus, the bone graft material can maintain activity only with a sequence of minimum amino acids on the surface while reducing the loss and side effects of the tissue growth factor caused by the physical incorporation and application of the drug, thus providing additional advantages to therapeutic effects.

In the present invention, all kinds and types of bone graft materials and scaffolds can be used if they are used in the technical field. Preferred examples of these bone graft materials and scaffolds include organism-derived bone mineral powders and porous blocks originated from autogeneous bone, bovine bone and porcine bone, synthetic hydroxyapatite powders and porous blocks, tricalcium phosphate powders and porous blocks, monocalcium phosphate powders and porous blocks, bone graft materials made of silicon dioxide (silica), bone-packing graft materials made of a mixture of silica and polymer, fine particles and porous scaffolds made of biocompatible polymers, including chitosan and polylactic acid, and titanium and three-dimensional scaffolds. In this respect, the surface of the bone graft materials and scaffolds is preferably modified so as to facilitate the adhesion of the active peptide to the surface. The scaffolds for tissue engineering applications according to the present invention include barrier membranes, tooth implants and the like which are used for the regeneration of periodontal bone.

Preferred examples of the barrier membranes include, but art not limited to, porous membranes made of polylactic acid, regeneraton membranes made of nanofibers of chitin or chitosan, and film-shaped barrier membranes made of chitin or chitosan. Also, as the implants, titanium implants are preferably used but are not limited thereto. In this respect, the surface of the implants is preferably modified by oxidation and nitrification so as to facilitate the adhesion of the active peptide to the surface.

The peptides according to the present invention are not more sensitive to *in vivo* enzymatic reactions than the tissue growth factor itself and have a lower *in vivo* immunogenicity. When the active peptides are immobilized on the surface of bone graft materials, scaffolds, barrier membranes or implants for tissue regeneration and used in surgical operations, the desired concentration of the active peptides can be locally present while showing activity, so that their therapeutic effects can be increased. Thus, the active peptides have suitable characteristics for the regeneration and repair of bone tissue and periodontal tissue.

The inventive peptides having free amino groups or cysteines at the N-terminal end are easy to immobilize on the surface of bone graft materials and scaffolds by crosslinkers. Crosslinkers suitable for use in the present invention include, but are not limited to, 1,4-bis-maleimidobutane (BMB), 1,11-bis-maleimido tetraethyleneglycol (BM[PEO]$_4$), 1-ethyl-3-[3-dimethyl aminopropyl] carbodiimide hydrochloride (EDC), succinimidyl-4-[N-maleimido methylcyclohexane-1-carboxy-[6-amidocaproate]] (SMCC) and sulfo-SMCC, succimidyl 6-[3-(2-pyridyldithio)-ropionamido] hexanoate] (SPDP) and sulfo-SPDP), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) and sulfo-MBS, succimidyl [4-(p-maleimidophenyl) butyrate] (SMPB) and sulfo-SMPB.

In addition, the peptides are chemically bound to the surface of bone graft materials and scaffolds so that they are immobilized on the surface in an amount of preferably 0.1-10 mg/cm^2, and more preferably 1-5 mg/cm^2.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 shows the results of electronic surface analysis of peptides immobilized on bone graft materials according to the present invention. In FIG. 1, (A) shows the results of electronic surface analysis of a bone graft material having no peptide immobilized on the surface, and (B) shows the results of electronic surface analysis of a bone graft material having sulfur-containing peptides immobilized on the surface.

FIG. 2 shows confocal laser scanning microscopic images showing the cell adhesion patterns of the inventive bone graft materials. In FIG. 2, (A) shows the adhesion pattern of cells to a bone graft surface having no immobilized peptides, and (B) and (C) show the adhesion patterns of cells on bone graft surfaces with immobilized peptides derived from BMP and bone sialoprotein, respectively.

FIG. 3 is a graphic diagram showing the results of quantitative analysis for the adhesion of cells on the inventive bone graft surface with immobilized peptides.

FIG. 4 is a photograph showing the results of Western blot measurement for the amount of bone tissue differentiation markers smad 1, 5, 8 proteins in cells collected after dispensing cells on the inventive bone graft material and culturing the dispensed cells for a given period of time (Con: a bone graft material with no immobilized peptide; BMP: a bone graft material having BMP-derived peptides immobilized thereon; and BSP: a bone graft material

having sialoprotein-derived peptides immobilized thereon).

FIG. 5 shows photographs showing the bone regeneration ability of the inventive bone graft materials at rabbit cranial defects. In FIG. 5, (A) shows the bone regeneration ability of a bone graft material (HA) with no immobilized peptide at rabbit cranial defects, and (B) shows the bone regeneration ability of the inventive bone graft material (HA) with immobilized peptides at rabbit cranial defects. New Bone represents bone produced by peptides immobilized on the surface of the bone graft material.

DETAILED DESCRIPTION OF THE INVENTION

Hereinafter, the present invention will be described in further detail by examples. It will however be obvious to a person skilled in the art that these examples are provided for illustrative purpose only and are not construed to limit the scope of the present invention.

Example 1: Immobilization of cell adhesive RGD peptides on bovine bone-derived bone mineral particles

Bovine bone-derived bone mineral particles were washed with ethanol under reduced pressure and then left to stand in a vacuum oven at 100 °C for 20 hours so as to remove impurities from the surface. The surface of the bone mineral particles was treated with a solution of 3-aminopropyl ethoxysilane (APTES) dissolved in hexane, followed by washing. This resulted in the formation of amine residues on the surface, to which crosslinker BMB was then added and bound. The crosslinker-bound bone mineral particles were allowed to react with peptides of SEQ ID NO: 1 and SEQ ID NO: 2 for 12 hours, followed by washing. This yielded the bone mineral particles having

the peptides immobilized on the surface.

__**Example 2:** Immobilization of cell adhesive RGD peptides on synthetic hydroxyapatite and tricalcium phosphate__

Bone graft powders of synthetic hydroxyapatite and tricalcium phosphate were washed with ethanol under reduced pressure and then left to stand in a vacuum oven at 100 °C so as to remove impurities from the surface. The surface of the bone mineral particles was treated with a solution of 3-aminopropyl ethoxysilane (APTES) in hexane, followed by washing. This resulted in the formation of amine residues on the surface, to which crosslinker BMB was then added and bound. The bone mineral particles with the bound crosslinker were allowed to react with peptides of SEQ ID NO: 1 and SEQ ID NO: 2 for 12 hours, followed by washing. This yielded the bone mineral particles having the peptides immobilized on the surface.

__**Example 3:** Immobilization of cell adhesive RGD peptides on bone graft material of chitosan__

A bone graft material of chitosan prepared in the form of a powdery or porous scaffold was added to 2 ml of phosphate buffer (pH 7.4) to hydrate the surface. To this solution, sulfo-SMCC as a crosslinker was added at a concentration of 5 mg/ml, and the mixture was stirred for 2 hours to introduce functional groups on the surface of the chitosan bone graft material. After 2 hours of reaction at ambient temperature, the chitosan bone graft material was washed and allowed to react with a solution 10 mg of a peptide of SEQ ID NO: 1 dissolved in 100 μl of phosphate buffer for 24 hours. Then, the reaction was washed, thus yielding the chitosan bone graft material with the peptide immobilized thereon.

__**Example 4:** Immobilization of cell adhesive RGD peptide on bone graft__

material on bone graft material of polylactic acid

A grafting powder or porous scaffold of polylactic acid were added to phosphate buffer (pH 4.7) to hydrate the surface, followed by reaction with 20 mg/ml of cystamine hydrochloride solution. To this solution, EDC was added dropwise to activate the carboxylic acid on the surface of the bone graft material. The mixture was reacted for 24 hours, washed, and allowed to react with 1 ml of dithiothreniol (DTT) solution (30 mg/ml) for 24 hours so as to introduce sulfhydryl groups onto the surface of the polylactic acid. The modified polylactic acid grafting material was mixed with a cell adhesive RGD peptide (SEQ ID NO: 1) so as to induce S-S bonds between the sulfhydryl groups of the bone grafting material and the peptides, thus immobilizing the peptides on the grafting material.

Example 5: Immobilization of tissue growth factor-derived peptides on bone mineral particles

For use as tissue growth factor-derived peptides in this Example, peptides were chemically synthesized by adding a CGG spacer to the N-terminal end of each of amino acid sequences of SEQ ID NO: 3 and SEQ ID NOS: 6-9, which contain the cell adhesion and activation domain of bone morphogenetic protein BMP-2 so as to introduce cysteine into the N-terminal end.

Bovine bone-derived bone mineral particles were washed with ethanol under reduced pressure and then left to stand in a vacuum oven at 100 °C for 20 hours so as to remove impurities from the surface. The surface of the bone mineral particles was treated with a solution of 3-aminopropyl ethoxysilane (APTES) in hexane, followed by washing. This resulted in the formation of amine residues on the surface of particles, to which sulfo-SMCC as a crosslinker was then added at a concentration of 5 mg/ml. This mixture was

stirred for 2 hours so as to introduce functional groups onto the surface of the bone graft material. After 2 hours of reaction at ambient temperature, the bone graft material was washed, and allowed to react with a solution of 10 mg of the peptides dissolved in 100 μl of phosphate buffer for 24 hours, followed by washing. This yielded the bone mineral particles with the peptides immobilized thereon.

Example 6: Immobilization of tissue growth factor-derived peptides on particles of synthetic bone graft material

In this Example, the same peptides as used in Example 5 used as tissue growth factor-derived peptides. As synthetic bone graft materials, mineral particles of synthetic hydroxyapatite and tricalcium phosphate were washed with ethanol under reduced pressure and stored in a vacuum oven at 100 °C for 20 hours so as to remove impurities from the surface. The surface of the particles was treated with a solution of 3-aminopropyl ethoxysilane (APTES) in hexane, followed by washing. This resulted in the formation of amine residues on the surface, to which 5 mg/ml of sulfo-SMCC as a crosslinker was added. The mixture was stirred for 2 hours to introduce functional groups onto the surface of the bone graft material. After 2 hours of reaction at ambient temperature, the bone graft material was washed, and allowed to react with a solution of 10 mg of the peptides dissolved in 100 μl of phosphate buffer for 24 hours, followed by washing. This yielded the bone graft particles with the tissue growth factor-derived peptides immobilized thereon.

Example 7: Immobilization of tissue growth factor-derived peptides on bone graft material and scaffold of chitosan

A bone graft material and scaffold made of chitosan was added to 2 ml of phosphate buffer (pH 7.4) so as to hydrate the surface, to which crosslinker

sulfo-SMCC was added at a concentration of 5 mg/ml. The mixture was stirred for 2 hours so as to introduce functional groups onto the surface of the chitosan bone graft material. After 2 hours of reaction at ambient temperature, the chitosan bone graft material was washed, and allowed to react with a solution of 10 mg of the tissue growth factor-derived peptide of Example 5 dissolved in 100 μl of phosphate buffer, followed by washing. This yielded the chitosan bone graft material and scaffold having the peptide immobilized thereon.

Example 8: Immobilization of tissue growth factor-derived peptide on bone graft material and scaffold of polylactic acid

A bone grafting powder or porous scaffold of polylactic acid was added to phosphate buffer (pH 4.7) to hydrate the surface and allowed to react with 20 mg/ml of cystamine hydrochloride solution. To the reaction mixture, crosslniker EDC was added dropwise to activate the carboxylic acids on the surface of the polylactic acid bone graft material. After 24 hours of reaction, the resulting material was washed, and allowed to react with 1 ml of dithiothreniol (DTT) solution (30 mg/ml) for 24 hours so as to introduce sulfhydryl groups onto the surface of the polylactic acid. The bone graft material was mixed with a tissue growth factor-derived peptide of SEQ ID NO: 8 having a CGG spacer bound thereto, so as to spontaneously induce a S-S bond between the bone graft material and the peptide, thus immobilizing the peptide on the bone graft material.

Example 9: Immobilization of bone sialoprotein-derived peptides on bone mineral particles

For use as bone sialoprotein-derived peptides in this Example, a peptide of SEQ ID NO: 15, a peptide including an active domain structure for the

induction of calcification, and a peptide of SEQ ID NO: 27 including a cell adhesion functional site, were chemically synthesized.

Bovine bone-derived bone mineral particles were washed with ethanol under reduced pressure and then left to stand in a vacuum oven at 100 °C for 20 hours so as to remove impurities from the surface. The surface of the bone mineral particles was treated with a solution of 3-aminopropyl ethoxysilane (APTES) in hexane, followed by washing. This resulted in the formation of amine residues on the surface, to which 5 mg/ml of crosslinker Sulfo-SMCC was then added. The mixture was stirred for 2 hours so as to functional groups onto the surface of the bone graft material. After reaction, the bone graft material was washed, and allowed to react with a solution of 10 mg of the bone sialoprotein-derived peptides dissolved in 100 µl of phosphate buffer for 24 hours, followed by washing. This yielded the bone mineral particles having the peptides immobilized thereon.

<u>**Example 10:** Immobilization of bone sialoprotein-derived peptides on synthetic bone graft particles</u>

In this Example, the same peptides as used in Example 9 were used. Hydroxyapatite and tricalcium phosphate mineral particles were washed with ethanol under reduced pressure and then left to stand in a vacuum oven at 100 °C for 20 hours so as to remove impurities from the surface. The surface of the particles was treated with a solution of 3-aminopropyl ethoxysilane (APTES) in hexane, followed by washing. This resulted in the formation of amine residues on the surface, to which 5 mg/ml of crosslinker sulfo-SMCC was then added. The mixture was stirred for 2 hours so as to introduce functional groups onto the surface of the bone graft material. After completion of the reaction, the bone graft material was washed, to which a solution of 10 mg of the same peptides as used in Example 9, which have been

dissolved in 100 μl of phosphate buffer, was added and allowed to react for 24 hours. The reaction product was washed, thus yielding the bone graft material having the peptides immobilized thereon.

Example 11: Immobilization of peptides containing adhesion and activation sites of bone sialoprotein on bone graft material of chitosan

In this Example, the same peptides as used in Example 9 were used. A bone graft material and scaffold of chitosan were added to 2 ml of phosphate buffer (pH 7.4) to hydrate the surface. To this solution, 5 mg/ml of crosslinker sulfo-SMCC was added and stirred for 2 hours to introduce functional groups onto the surface of bone graft material. After completion of the reaction, the chitosan bone graft material was washed, to which a solution of 10 mg of the peptides dissolved in 100 μl of phosphate buffer was added and allowed to react for 24 hours, followed by washing. This yielded the chitosan bone graft material and scaffold having the peptides immobilized thereon.

Example 12: Immobilization of peptides containing adhesion and activation sites of bone sialoprotein on bone graft material and scaffold of polylactic acid

In this Example, the same peptides as used in Example 9 were used. A bone graft material and scaffold of polylactic acid were added to phosphate buffer (pH 4.7) so as to hydrate the surface, and then allowed to react with 20 mg/ml of cystamine hydrochloride solution. To the reaction mixture, crosslinker EDAC was added dropwise to activate the carboxylic acids on the surface of the polylactic acid. After 24 hours of reaction, the reaction product was washed, to which 1 ml of DTT solution (30 mg/ml) was added and allowed to react for 24 hours so as to introduce sulfhydryl groups onto the surfaces of the bone graft material and the scaffold. The bone graft material and the scaffold were mixed with the peptides so as to spontaneously induce S-S bonds between

the bond graft material and the peptides, thus immobilizing the peptides on the bone graft material.

Example 13: Immobilization of osteogenesis-promoting peptides on barrier membrane of chitosan

A barrier membrane of chitosan was added to 2 ml of phosphate buffer (pH 7.4) to hydrate the surface of the barrier membrane. To the solution, 5 mg/ml of crosslinker sulfo-SMCC was added and the mixture was stirred for 2 hours so as to introduce functional groups onto the surface of the barrier membrane. After completion of the reaction, the barrier membrane was washed, to which a solution of 5 ml of each of a cell adhesion peptide having SEQ ID NO: 1, a BMP-2-derived peptide used in Example 9, and a bone sialoprotein-derived peptide used in Example 9, which has been dissolved in 100 μl of phosphate buffer, was added and allowed to react for 24 hours. After washing, the barrier membrane having the peptides immobilized thereon was obtained.

Example 14: Immobilization of osteogenesis-promoting peptides on a barrier membrane of polylactic acid

A barrier membrane of polylactic acid was added to phosphate buffer (pH 4.7) so as to hydrate the surface, and is allowed to 20 mg/ml of cystamine hydrochloride solution. To the reaction mixture, crosslinker EDC was added dropwise to activate the carboxylic acids on the surface of the polylactic acid. After 24 hours of reaction, the barrier membrane was washed, to which 1 ml of DTT solution (30 mg/ml) was added and allowed to react for 24 hours so as to introduce sulfhydryl groups onto the surface of the barrier membrane. The resulting barrier membrane was mixed with each of a cell adhesion peptide of SEQ ID NO: 1, a BMP-2-derived peptide used in Example 5 and a bone sialoprotein-derived peptide used in Example 9 so as to spontaneously induce

S-S bonds between the barrier membrane and the peptides, thus immobilizing the peptides on the barrier membrane.

Example 15: Immobilization of osteogenesis-promoting peptide on titanium implant

The surface of an implant made of titanium was treated with nitrogen plasma so as to form amine groups on the surface. To the implant, 5 mg/ml of crosslinker sulfo-SMCC was added and stirred for 2 hours so as to introduce functional groups onto the surface. After completion of the reaction, the implant was washed, to which a solution of each of 5 ml of a cell adhesion peptide having SEQ ID NO: 1, a BMP-2-derived peptide used in Example 5 and a bone sialoprotein-derived peptide used in Example 9, which has been dissolved in 100 μl of phosphate buffer, was added and allowed to react for 24 hours. The resulting implant was washed, thus the obtaining the implant having the peptides immobilized thereon.

Test Example 1: Analysis of surface of bone graft materials according to the present invention

In order to analyze the surface of each of the peptide-immobilized bone graft materials prepared in Examples 1-12, the bone graft materials were fixed with 2% glutaraldehyde solution. The fixed bone graft materials were treated with 1% osmium tetroxide solution, followed by washing, dewatering and drying.

The surface of the prepared bone graft materials was analyzed by an XPS method which determines the presence or absence of bonds by identifying elements immobilized on the surface of a substance. In this respect, the presence or absence of bonds were determined depending on the presence or absence of sulfur since there are disulfide bonds between the bone graft

material and the peptides immobilized on the bone graft material according to the present invention.

FIG. 1 shows the results of analysis of peptides immobilized on a bone graft material of chitosan according to the present invention. In FIG. 1, (A) shows the surface of a bone graft material made of chitosan, which has not been modified with peptides, and (B) shows a bone graft material having a sulfur-containing peptide immobilized on the surface. As shown in FIG. 1, the presence of sulfur on the surface of the peptide-immobilized bone graft material was observed, suggesting that the peptides were immobilized. Furthermore, the content of sulfur in the peptide-immobilized bone graft material was measured in order to determine the immobilization rate of the peptide in the total surface area of the bone graft material. As a result, as shown in Table 1 below, sulfur was not detected in the chitosan with no peptide whereas 8.66% of sulfur was detected in the peptide-immobilized chitosan.

Table 1

Element	O (%)	N (%)	C (%)	S (%)	O/C	N/C
Chitosan with no peptide	31.83	6.18	61.99	0	0.513	0.0997
Peptide immobilized chitosan	32.33	2.96	60.05	8.66	0.605	0.033

<u>**Test Example 2:** Test of cell adhesion of bone graft materials according to the present invention</u>

Osteoblasts ((MC3T3 cell line) were inoculated on the peptide-immobilized bone graft materials prepared in Examples 3, 7 and 11 and then cultured for each of 4 hours and 1 day. The bone graft materials with the cultured osteoblasts were fixed with 2% glutaraldehyde solution. The fixed bone graft materials were added with a fluorescent-labeled phalloidin solution treated

with 1% triton X-100, thus staining the cytoplasm. Then, after the samples were washed and fixed, the cells adhered to the bone graft materials were observed with a confocal laser scanning microscope (FIG. 2).

In FIG. 2, (A) shows the cell adhesion to the bone graft material with no peptide, and (B) and (C) show the cell adhesion to the bone graft materials on which the BMP-derived peptide and the bone sialoprotein-derived peptide have been immobilized, respectively. As a result, for the bone graft material with no immobilized peptide, the spherical and unstable adhesion of the cells was observed, whereas on the surfaces of the bone graft materials with the BMP- and bone sialoprotein-derived peptides, the stable adhesion of the cells (including the elongation of the cytoplasm in most of the cells after 4 hours of the cell culture) was observed.

FIG. 3 shows the results of quantitative analysis for the cell adhesion. As shown in FIG. 3, the chitosan bone graft materials modified with the peptides showed a remarkable increase in the adhesion of the cells as compared to the chitosan bone graft material with no immobilized peptide, and this increase was proportional to the amount of the immobilized peptides up to any concentration.

<u>Test Example 3:</u> <u>Expression of differentiation marker proteins in osteoblasts cultured on surface of peptide-immobilized bone graft material according to the present invention</u>

In order to determine the expression of differentiation marker proteins in osteoblasts cultured on the surface of the peptide-immobilized bone graft material according to the present invention, the expression level of differentiation marker proteins smad 1, 5 and 8 was analyzed by Western blot.

Osteoblasts were inoculated on the surfaces of the bone graft material and the peptide-immobilized bone graft material and then cultured for 2 weeks. After culturing, total protein in the cells was extracted, and quantified by measuring the absorbance at 280 nm. 2 μl of the protein solution (1 mg/ml) was taken and electrophoresed on acrylamide gel, followed by reaction with an antibody to differentiation marker proteins smad 1, 5 and 8. Then, the protein solution was allowed to react with a labeled secondary antibody, and protein bands appearing by the development of the gel were observed and their density was measured (FIG. 4).

As a result, as shown in FIG. 4, the expression of the smad proteins cultured on the surface of the peptide-immobilized bone graft material was significantly increased as compared to the case of the cells cultured on the bone graft material with no immobilized peptide. This suggests that the cells grown on the surface of the bone graft material having the tissue growth factor-derived peptide immobilized on the surface are differentiated into bone tissue in a facilitated manner.

Test Example 4: Effect of peptide-immobilized bone graft material on rabbit cranial regeneration

The peptide-immobilized bone graft materials prepared in Examples 1-5 were grafted in rabbit cranial circular defects in order to examine their bone regeneration ability. At the cranial sites of anesthetized rabbits, circular bone defects with a diameter of 8 mm were formed. The bone graft material and the peptide-immobilized bone graft materials were grafted into the bone defects at an amount of 50 mg/defect, and the bone membrane and the skin were double sutured to each other. At 2 weeks after the grafting, the animals were sacrificed, and samples collected from the animals were fixed in formalin solution and then the tissue was embedded so as to prepare samples having a

thickness of 20 μm. The prepared samples were stained with basic fuchsine and toluidine blue, thus preparing non-decalcified samples. The prepared samples were photographed with an optical microscope.

FIG. 5 shows the bone regeneration effect of the peptide-immobilized bone graft materials. As shown in FIG. 5, the inventive bone graft materials having the osteogenesis-promoting peptide adhered to the surface, which have been applied to the rabbit cranial defects (B), showed remarkable bone regeneration ability within 2 weeks as compared to the bone graft material with no peptide (A).

Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof. Those skilled in the art will appreciate that simple modifications, variations and additions to the present invention are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

INDUSTRIAL APPLICABILITY

As described above, the present invention provides the bone graft material and scaffold having a surface immobilized with the cell adhesion-inducing peptide and/or the tissue growth factor-derived peptide, which can achieve the desired tissue regeneration effect even at the low concentration dose level.

The inventive bone graft material and the scaffold for tissue engineering applications, have the osteogenesis-promoting peptides immobilized on the

surface, can promote the adhesion of cells and the differentiation of cells into bone tissue, and can prevent rapid degradation of a tissue growth factor caused by its simple incorporation according to the prior art, and side effects resulting from its leakage into the body. Moreover, they allow a great reduction in the costs caused by applying a large amount of the tissue growth factor to increase its local concentration.